Description

AN ASSAY CHIP, AND USES OF SAID ASSAY CHIP TO DETERMINE MOLECULAR STRUCTURES
AND FUNCTIONS

The invention relates to an assay chip for investigation of
the functionality of membrane proteins and their interactions
with molecules. Further, the invention relates to a process
for analyzing the functionality of non-lipid molecules, such
as a protein, being integrated in a fluid biological
effective layer. Furthermore, the invention relates to the
use of said assay chip.

Detailed knowledge about protein structures and their related
functions is a key to understand molecular processes of life.
Due to the powerful molecular cloning and gene expression
technologies proteins for the purpose of analytical
investigations can be produced in sufficient amounts.
Proteomics is presently a very active field in life sciences
research. The final aim is to achieve a comprehensive
understanding of structure and function of proteins. This
knowledge is a prerequisite for a rational design of new
drugs. G-protein coupled receptors (GPCR) constitute the
largest subgroup of cell membrane receptors and about half of
them are considered to be targets for drugs. As for an
example, the actual structure of bovine rhodopsin, a model
for GPCR proteins, could recently be determined at a 2.8 Å
resolution. GPCRs have a glycosylated N-terminal ligand
binding site, seven trans-membrane helices and an
intracellular G-protein binding domain. Ligand binding on the
extracelluar part induces conformational changes in the
trans-membrane helices bundle of the receptor protein
resulting in a dissociation of the hetero-trimeric G-protein
which is bound to the intracellular part of the GPCR into a
α- and a βγ-subunit. G-protein are anchored covalently to the

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lipid bilayer and are currently classified in four families
according to the nature of the α-subunits which interact with
different target membrane proteins like enzymes or ion
channels. After the dissociation of the G-protein the α-
5   subunit laterally diffuse within the lipid bilayer and bind
to the target protein and activate it.

Therefore, the composition and the fluidity of the lipid
bilayer are in any type of functional analysis and screening
10   process critical issues, especially of high interest when
considering an economic process for screening purposes.

Arrays of immobilized GPCRs in micro-spots have recently been
used to investigate compounds which specifically bind to this
15   membrane protein. Such high throughput technologies allow a
screening across or within receptor families and may be
suitable for ligand fishing for orphan GPCRs. The biological
response of the current about 140 orphan GPCR receptors are
unknown which make it difficult to identity ligands as
20   potentially useful drugs.

Deorphanization of non-olefactory GPCRs is currently a focus
in the pharmaceutical industry and recently receptors with
potential functions related to cancer and diabetes has been
25   identified. The success in finding new binding compounds
depends strongly on full functionality of the target protein
as it is the case in the living cell. Allosteric sites on
GPCRs which do not overlap with the binding sites for the
natural agonist have a number of theoretical advantages over
30   agonist binding sites for drugs such as saturability and high
tissue specificity. Thus, allosteric sites are attractive
targets sites for drugs which modulate receptor functions by
increase or partially decreasing its activity. The binding of
allosteric ligand or modulating proteins can result in
35   membrane protein complexes consisting of several components.
Again, for the investigation of such interactions highly
sensitive functional assay systems are required in which the

target membrane protein has to be fully mobile within the
lipid bilayer membrane and well accessible.

In almost all cases of the *in vitro* assay systems as outlined
5   below, biomembranes of unknown composition are immobilized on
solid supports which often leads to a restricted fluidity of
the lipid bilayer and a structural disturbance of the
membrane protein. For that reason, these circumstances are
greatly undesired in broad assay processing campagnes since
10   for the drug screening processing the native functions of the
proteins are potentially highly disturbed.

A second important class of membrane proteins are ion
channels. The voltage-gated sodium channels of eukaryotes are
15   complex membrane proteins composed of more than 2000 amino
acids residues. The bacterial sodium channel is much simpler
consisting of one-domain of 34 kD and it can be expressed at
high rates and is therefore useful for assay technology
development. High throughput screening assays use lipid
20   bilayer membranes associated sensitive reporter molecules or
fusion constructs consisting of the green fluorescence
protein and the ion channel. By the use of the proposed
highly sophisticated assay systems, a deeper insight into the
function of membrane proteins of interest will be achieved.
25   Both, academic research and drug discovery will profit from
this knowledge.

Cell-based assays are well suited to monitor the biological
response of GPCRs in their natural environment and are widely
30   used to identify lead compounds in the drug discovery
process. In order to understand molecular mechanisms, *in
vitro* tests are required where the number of involved
components is reduced. The functionality of membrane proteins
in vivo depends on many factors: the composition of the lipid
35   bilayer membrane, biological activation reactions such as
phosphorylation or the intracellular Ca^{++} concentration. The
development of robust, functional biomimetic *in vitro* tests

for membrane proteins is a demanding, highly
interdisciplinary field. The first step is the reconstitution
of the purified expressed recombinant proteins into lipid
bilayer membranes, in order to bring them in the functional
5   form. Vesicles consisting of lipid bilayers are commonly used
to keep membrane proteins active in buffer solutions. Binding
of ligands to proteins present in vesicles has been monitored
when they are bound on sensor surfaces using surface plasmon
resonance as the detection method. However, such vesicle
10   preparations may require too high quantities of the protein
to achieve a sufficiently high sensitivity in the
measurements and they are not well suited to investigate many
functional aspects. Therefore, several types of hybrid
systems have been developed where planar lipid bilayer
15   membranes are immobilized on solid supports.

Free standing membranes which span a hole in a Teflon support
appear black. Such black lipid bilayer membranes are
difficult to prepare and are extremely fragile since the
20   lipid bilayer of 4 nm thickness has to span holes in the
range of 200 µm to 1 mm. For research experiments, this
limitation may be acceptable, however, for practical
applications in the drug discovery stability of the assay
system is a major issue. Therefore, since about 20 years
25   techniques have been developed using supports in order to
achieve a higher stability. Membrane proteins have been
immobilized by fusion of lipid vesicles to supported lipid
bilayers or directly to SiO_2. Planar bilayer formation from
vesicle adsorbed to hydrophilic surfaces like mica could be
30   visualized.

The changed physical properties of lipid bilayers when
supported, however, strongly affect the function of the
embedded membrane protein. In order to overcome these
35   problems, lipid bilayer membranes have been immobilized on
surfaces via tethers. The resulting cleft between the lipid
bilayer membrane and the solid support mimics the

intracellular space and the desired functionality of trans-
membrane proteins is partially retained. Ligand binding to
membrane proteins in the lipid bilayer has been monitored
using fluorescence detection. Impedance was measured to

5   determine the function of supported ion channels as well. The
conductance of supported lipid bilayer membranes containing
an iono-phore changes upon binding of ligands. This effect
has been utilized to develop membrane protein based
biosensors.

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The advantages of a hybrid system are a gain in stability and
the feasible preparation procedure. The formation of
supported planar lipid bilayer membranes by fusion of
vesicles with supported phospholipid monolayers has been

15   established. For optical detection of ligand binding lipid
bilayer membranes have been prepared on alkylated
carboxylated dextrane polymer which has been immobilized on
sensor surfaces in a high density. In a study using
micropatterned surfaces it has been shown that hydrophilic-

20   hydrophobic edges promote vesicle fusion. Furthermore, the
fusogenic effects of short-chained alcohols, Ca^{++}-ions and
PEG are known in the art. These studies show that vesicle
composition, fusogenic agents, hydrophilicity, topographic
and chemical properties of the surface and temperature are

25   the most important factors influencing the formation of lipid
bilayer membranes from vesicles adsorbed to solid supports.

Supported tethered lipid bilayer membranes as achieved
according to the methods described above have also some major

30   disadvantages. The composition of the fluid in the reservoir
between the lipid bilayer membrane and the support can hardly
be controlled and the fluidity of the immobilized lipid
bilayer membrane is restricted. This constitutes a tremendous
drawback since the functionality of membrane proteins is

35   related to the fluidity of the lipid bilayer and their
mobility therein. Additionally, the incorporation of membrane
proteins with large trans-membrane loops – the acetylcholine

receptor for instance has a 5 nm large intracellular part -
are impeded since the intracellular space between the lipid
bilayer and the support gained by a tether are too small for
larger trans-membrane loops.

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Furthermore, only one side of the tethered lipid bilayer
membrane is accessible which makes it difficult to explore
trans-membrane changes or transport of molecules. The non-
native environment may result in an impairment of the
10   functionality and in screenings physiological receptor
functions may not be detected.

In the following the different membrane types like prepared
lipid bilayers with a predefined composition, biomembranes of
15   cellular origin and mostly unknown composition or functional
layers of supramolecular assemblies are generally called
biological effective layer.

It is therefore a specific aim of the invention to provide a
20   device with a biological effective layer as a membrane having
high and reliable stability in a manner that its fluidity is
sustained in order to integrate a non-lipid molecule into
said layer preserving its full biological functionality.

25   This aim will be achieved according to the invention by an
assay chip for investigation of the functionality of non-
lipid molecules and their interactions with molecules,
comprising:

30   a) a nanopore substrate having a plurality of nanopores;
          b) a suitable substantially planar support layer deposited
             on said nanopores substrate having a plurality of
             nanopores corresponding with said nanopore substrate;
          c) a biological effective layer being capable to host at
35   least a non-lipid molecule or functional molecule,
             deposited on said support layer and covering the
             plurality of nanopores, resulting in accessible

nanopores from both sides of the biological effective
layer for measurements.

This assay chip offers an array of nanopores of macroscopic
5   lateral dimension therefore providing both, supporting area
to stabilize the biological effective layers (defined as a
layer that preserves the full functionality of the non-lipid
molecule hosted therein), such as a lipid bilayer membrane,
and pores in a high density in which the biological effective
10   layer remains fully fluid. This assay chip therefore offers a
versatile system for various applications, like drug
screening, functional protein analysis, toxicity analysis and
the like. Due to the tiny thickness of the Si_3N_4-layer the
respective Si_3N_4-membrane with the nanopores is also
15   extremely thin and due to the applied fabrication technology
these Si_3N_4-membranes are mechanically stable. The given
aspect ratio of the pore diameter to thickness of the
nanopore array allows an un-impeded diffusion of
macromolecules to both, the lipid layer membrane and to the
20   non-lipid molecule, such as membrane proteins, integrated
therein. Further, the mechanically stabilized biological
effective layer (that means the solid support layer being the
Si_3N_4-membrane with the nanopores and the biological
effective layer immobilized thereupon) offers free access
25   from both sides of the biological effective layer what allows
the investigation of complex interactions of molecules, such
as natural ligands or the interaction with artificial
effector molecules (such as drugs) with functional integrated
membrane proteins and to elucidate the mechanism of signal
30   transduction. Due to the accessibility from both sides, the
transport of ions, molecules and particles through the
biological effective layer by transporter proteins can be
investigated in a micro-chamber system, i.e. in a two-
compartment system. Surface patterning and microspotting
35   technologies will allow to address specific nanopore arrays.
Furthermore, the membrane proteins are sterically not impeded
due to the preservation of their mobility and therefore can

directly be investigated on their response to allosteric
effects what is crucial for the development of new drugs with
GCPRs as the target. The total surface area being
nanostructured is in a range that on the one hand a

5   sufficient amount of membrane protein molecules is present in
order to enable the use of macroscopic methods for detection
of a distinct binding by means of fluorescence or other
sensitive detection methods. On the other hand the amount of
precious membrane proteins and/or binding compounds is

10 comparably low for the achievement of the desired screening
process.

This assay chip with the nanostructured silicon nitride
membrane supports the biological effective layer and thus

15 bio-mimicks the cytoskeleton.

Materials for a suitable support layer are silicon nitride
(Si_3N_4) or silicon oxide (SiO_2) and the nanopore substrate is
potentially of silicon and carbon containing materials but

20 also a polymer, a metal, a dielectrica, a glass or a ceramic.
Suitable is insofar meant as a definition that the properties
of the support material do allow adhesion of the lipid layer
that is supposed to be supported by the support layer.
Additionally, it should be pointed out that already the

25 support layer may have chemical and topographical properties
that promote the fusion of the lipid layer on the support
layer.

In order to improve or induce the formation and/or fusion

30 and/or immobilization of the fluid lipid bilayers on the
support layer to a desired extent, the surface of the support
layer may be modified resulting in a promotion layer, i.e.
using chemically activated hydrophobic or hydrophilic silanes
or other components as well as modifications of physical

35 nature such as topographical or electric modifications. This
promotion layer may be designed according to the properties

of the lipid bilayer to be supported and according to the
mechanism responsible for the formation of the lipid bilayer.

With respect to the stability and to the free diffusion of
5   macro-molecules the thickness of the support layer in the
nanopore array sections and the diameter of the nanopores may
be chosen in order to result with an aspect ratio in the
range of 0.25 to 5. In general, one nanopore array section
comprises nanopores each having a diameter in the range of 50
10 to 2000 nm, preferably 100 to 2000 nm and the nanopore array
sections have a thickness in the range of 50 to 2000 nm. The
area of the nanopore array sections have an area in the range
of 1×10^{-6} mm^2 to 1 mm^2. For example, a pore diameter of 200
nm requires the support layer to be 400 nm thick for matching
15 an aspect ratio of 2. Preferably, the aspect ratio may be in
the range of 0.75 to 2. Thereby, very well suited pore
diameters may range within an interval of 100 to 400 nm. This
size offers an excellent compromise between the mechanical
stability of the support layer and the biologically effective
20 layer as well as it preserves the fluidity of the liquid
bilayer membrane and the full functionality of the integrated
membrane proteins. Further, the number of membrane proteins
required for current macroscopic detection methods is limited
to an economically reasonable extent.
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With respect to achieve an efficient area of biological
effective layer that meets a proper relationship between the
area of the free standing lipid bilayer membranes in the
nanopores and the supported lipid bilayer membrane area, said
30 pores having a distance from each other in the range of 0.5
to 5-times, preferably 0.8 to 2-times of their diameter.

In practise, the biological effective layer may be isolated
from a natural source which essentially are prokaryotic or
35 eukaryotic cells as well as be a lipid bilayer prepared from
lipid vesicles and later fusion of them.

The lipid component for the reconstituted bilayer can be
selected from a group containing a phosholipid, a
cardiolipid, a lysophospholipid, a ceramide, a ganglioside, a
cerebroside, a glycolipid, a steroid, and a sphingolipid.

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The biological effective layer comprise at least one non-
lipid and/or functional molecule, whereby the non-lipid
molecule can be selected from a group containing a protein, a
polypeptide, a peptide or a synthetic compound (for instance
10   with a biomimetic function); the protein can be an enzyme, a
transport protein, a structural protein, a receptor, a
cytokine, a hormome, a toxin, an inhibitor or a chaperone.
These non-lipid molecules may be isolated or purified from a
natural source like cells of eukaryotes or prokaryotes.
15   Furthermore, the non-lipid molecule may be a synthetic
compound.

The biological effective layer comprise at least one non-
lipid and/or functional molecule, whereby the functional
20   molecule may be from artificial origin like recombinant DNA
or RNA technologies.

The biological effective layer may be formed with at least
one intact living cell. Depending on the actual geometry of
25   the nanopore array more than one cell may be used. The cells
can be embededed into a biological effective layer which was
deposited onto the nanopore array beforehand. If no such
layer has been deposited the cells represent the biological
effective layer on their own.

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A suitable procedure for the investigation of the
functionality of non-lipid molecules or functional molecule,
being integrated in a biological effective layer in such an
assay chip, according to the invention, comprises the steps
35   of:
     a) applying a fluid containing a molecule to one side of
        the fluid biological effective layer in order to allow

        the binding of the molecule to interact with the non-
        lipid molecule;

    b) monitoring the response of the non-lipid molecule
        induced by effector binding and/or the interacting of
5   binding molecules in the fluid biological effective
        layer by measuring physical or chemical changes on the
        cis- or trans-side of the assay chip.

    Therefore, the above-mentioned assay chip can be used
10 according to the invention in a drug discovery process with
    respect of the full functionality of membrane proteins in
    response to potential drug compounds to be screened. The
    binding can either be obeserved at the cis-side of the
    biological effective layer as e.g. fluorescence quenching or
15 at the trans-side measuring changes in pH, K^+, Na^+
    concentrations, measuring radioactivity or dyes in small
    volumes.

    The following description will explain some of the preferred
20 embodiments not limiting the invention and will quote the
    following drawings that are used to illustrate the present
    invention. Therefore, a brief description of the drawing is
    given below.

25 Figure 1   is a schematic view of the biological mechanisms
               occurring in fluid lipid bilayer

    Figure 2   is a schematic view on the design of a assay chip
               with a nanopore array and its dimension in a
30   distinct embodiment according to the invention; and

    Figure 3   is a schematic view on the manufacturing process of
               a assay chip with a nanopore array according to an
               embodiment of the invention.

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    According to figure 1, a portion of a assay chip 2 is shown
    to illustrate the biological function of a membrane protein

3. This assay chip 2 is the essential pre-requisite for the investigation of binding activities of the membrane proteins 3 which now combines both, the advantages of the supported 4 and of the free standing lipid bilayer membrane 5 as

5   discussed above. The assay chip 2 comprises an array substrate 28 and a 300 nm thick silicon nitride layer 6 having sections in form of arrays 7 of pores 8 of diameters in the range of 50 nm and 2 μm. This supporting silicon nitride layer 6 is chemically modified with a support

10   promotion layer 9 using activated hydrophobic silanes. The planar lipid bilayer membrane 4 on the surface of the supporting layer 6 or the support promotion layer 9, respectively, can be formed in various ways. In this embodiment, the hydrophobic chains 10 of a lipid vesicles are

15   interacting with the hydrophobic support promotion layer 9 forming spontaneously a bilayer 4. In a second step lipid vesicles 11 with integral membrane proteins 3 are added and interact with the immobilized lipid bilayer 4. Fusion 12 of the vesicles 11 adhering to the support promotion layer 9 and

20   in the second step to the formed bilayer can occur spontaneously at suitable conditions. Otherwise, fusion will be induced using fusogens. The fusion process can be monitored using optical, fluorescence and scanning probe microscopy or electrochemical methods.

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When a continuous lipid bilayer membrane 4 without defects is achieved with neat lipid vesicles 11, lipid bilayer membranes with commercially available Na^+ and Ca^{++} ionophores can be investigated. In a second phase membrane proteins are

30   investigated as specified below. Alternatively, solubilized membrane proteins 3 can be incorporated directly into the prepared lipid bilayer membrane 4 on the nanopore array sections 7. Membrane proteins 3 are expected to gather preferably in the free standing regions of the lipid bilayer

35   5 in the nanopores 8.

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The proposed concept allows to prepare membrane proteins 3 in
vesicle suspensions 11 in a first step and to add the
vesicles to the prepared lipid bilayer membranes 4 in a
second step 12. The resulting free standing lipid bilayer
5   membrane 4 has a sufficiently high mechanical stability
concomitant with a long-range fluidity which is required to
achieve full functionality of trans-membrane protein
preparations.

10 The assay chip 2 is used to investigate the functionality of
trans-membrane proteins 3 like GPCRs and their interactions
with further membrane proteins like ion channels 13. To
illustrate the importance of a fluid lipid bilayer for
membrane protein activity in general the function of GPCRs
15 are outlined in details. After binding of the natural ligand
14 to its binding site 15 which shall be represented by arrow
16 the GPCR get an catalytic activity resulting in a
dissociation 17 of the specific trimeric G-protein molecule
18 in a α- 19 and a βγ 20 subunit when it is bound to this
20 membrane protein 3 (arrow 21) on the other side of the lipid
bilayer membrane 4. The subunits 19 and 20 of the G-proteins
18 are covalently coupled via a lipid anchor to the lipid
bilayer membrane 4 and can laterally diffuse within the lipid
bilayer membrane 4 to the target membrane protein 13.
25 Additionally, the effect of docking an allosteric effector
molecule 22 to an allosteric site 23 of the membrane protein
3 (represented by arrow 24) can be monitored on its effect
modulating the receptor function by way as an enhancer,
agonist and antagonist. The induced structural changes in the
30 membrane protein 13 result in a varity of biochemical
reactions such as phosphorylation, c-AMP generation as
depending on the type of the α-subunit 19. In this specific
embodiment the target protein 13 is an ion channel and
transport of K^+-ions with high specificity through the lipid
35 bilayer 4 will occur which is monitored as fluorescence of a
potent K^+-specific crown ether indicator.

In this embodiment, the nanopore has a width of about 140 nm
that makes it clear to the person skilled in the art that the
membrane protein 3 is illustrated tremendously enlarged which
allows the exemplarily description of the biological

5   mechanisms. By an arrow 25 shall be indicated that the
membrane protein 3 retains its flexibility in moving along
the lipid bilayer 4 within the free standing pore region 5.
In reality the membrane proteins 3 have a size of only a few
nm that means that a number of a few membrane proteins 3 will

10 be hosted in the same pore region allowing an average free
lateral movement in the range of a large multiple of its
dimensions. The aspect ratio which shall be in this example
in the range of 1 thereby enables even larger molecules 26 to
diffuse un-impeded (arrow 27) into the free standing pore

15 region 5 and actually arrive at the receptor sites of the
membrane protein 3 integrated in the lipid bilayer 4.

Figure 2 depicts in a schematic way the design of a assay
chip 2 which comprises in this embodiment a array substrate

20 28 of 100 mm^2 total area having a 300 nm thin silicon nitride
layer 6 with the actual nanopore array 7. The size of the
silicon nitride membrane section 29 having the actual
nanopore arrays 7 is about 1 mm^2. A nanopore array section 7
of 400 x 400 μm comprises nanopores 8 having diameters in the

25 range of 50 to 2000 nm (indicated at 30). The distance of the
nanopores 8 to each other (the pitch) is chosen to be in the
range of their diameter 31. This guarantees both a sufficient
stability of the lipid bilayer 4 on the suitable support
layer 6 and a comparably high molecule density of membrane

30 proteins 3 and the compounds (14, 22) to be screened
diminishing utterly the amount of membrane proteins 3 and the
compounds to be screened as well. To support this argument, a
simple estimation is given below: Nanopores are disposed over
an area of 400 x 400 μm corresponds to 0.16 mm^2 having a

35 diameter of 125 nm and the same distance to each other. That
leads to 2 x 10^6 pores on said area. Assuming that in each
nanopore 8 a number of 10 membrane proteins molecules 3 of

about 5 nm diameter will be hosted leads to a total amount of
2×10^7 membrane protein molecules or $3 \ 10^{-17}$ mol and a low
filling factor of 0.2%. Depending on the molecular weight of
the respective membrane molecule, assuming a molecule having

5   a molecular weight of 30 kD, the required amount of the
respective molecule is in the range of 1 pg per assay chip.
An individual ion channel give a current of about 1 pA which
correspondes to 10^7 ions/sec. The turnover of all ion
channels in 100 sec will be about 10^{-7} mol ions in an

10 estimated liquid volume of about 10 µl which corresponds to a
concentration of about 1 millimolar. The activity of many
other membrane proteins will be lower, but the concentration
of the compound to be detected in the mentioned small volume
will be in the range of milli- to micromolar. This estimation

15 apparently discloses that very tiny amounts of the respective
molecules are required by using this assay chip design
thereby the quantity is still sufficient to monitor
concentration changes by detection methods on a macroscopic
level, such as fluorescence and impedance. These tiny amounts

20 of the respective molecules to be analysed towards its
functionality and reaction on or as a natural ligand binding
molecule and/or an allosteric effector molecule let the
vision to appear realistic that this assay chip is absolutely
the currently best choice for any type of drug discovery or

25 screening process.

Figure 3 schematically shows a process for manufacturing the
nanopores in order to achieve a chip 32 comprising the
substrate 28 and the support layer 6 with the nanopores 8.

30 First, the nanopores 8 are replicated by hot embossing
technique: A stamp 33 (figur 3A) is pressed into PMMA 34
(molecular weight 25 kg/mol) spin-coated to a thickness of
330 nm on a Si_3N_4 (260 nm)/Si (300 to 360 µm)/Si_3N_4 (260
nm)/Cr (40 nm) substrate 35. A silicon master (square silicon

35 pieces with side length 13.5 mm to be used as an embossing
stamp) were fabricated with a Leica LION-LV1 electron beam
writer using a scanned electron beam to write pore arrays in

PMMA resists 34. Here the pore diameter is defined by the aperture size and the defocus of the electron beam, and the pore period is defined by the scanning step size. The final steps of the master fabrication include Cr lift-off to
5   produce a dot array mask and them RIE of silicon dioxide to produce pillar arrays in the stamp with a height of 130 nm. The Si_3N_4 on the reverse side of the substrate is pre-structured with square openings (side length: 1 x 1 mm) to be used as a mask for the final Si_3N_4-membrane production step
10   (figure 3B). The Si_3N_4-layer on the front side of the substrate includes alignment marks (13.5 x 13.5 mm frame) to align the embossing master so that the nanopores coincide with the Si_3N_4-membranes, and break lines (10 x 10 mm frame) for removal of the final 10 x 10 mm chip form the wafer which
15   allows improved handling. Following embossing, PMMA residual layer is removed using an O_2 plasma and the pores transferred into the Cr layer with a Cl_2/CO_2 plasma (figure 3C). The PMMA mask is then removed with O_2 plasma before transferring the pores into the Si_3N_4 with a CHF_3/O_2 plasma. The Si is etched
20   with a KOH bath at 70°C to open the backside of the silicon nitride membranes (figure 3D) and the Cr mask is removed with Cl_2/CO_2 etch to give the final chip (figure 3E).

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